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Jonathan E. Markham

University of Nebraska-Lincoln, [jmarkham3@unl.edu](mailto:jmarkham3@unl.edu)

Nicholas J. Kruger

University of Oxford, [nick.kruger@plants.ox.ac.uk](mailto:nick.kruger@plants.ox.ac.uk)

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# Kinetic properties of bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from spinach leaves

Jonathan E. Markham and Nicholas J. Kruger

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK

Corresponding author – N. J. Kruger, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK; fax 44 1865 275074, tel 44 1865 275000, email [nick.kruger@plants.ox.ac.uk](mailto:nick.kruger@plants.ox.ac.uk)

## Abstract

A cDNA encoding 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase was isolated from a *Spinacia oleracea* leaf library and used to express a recombinant enzyme in *Escherichia coli* and *Spodoptera frugiperda* cells. The insoluble protein expressed in *E. coli* was purified and used to raise antibodies. Western blot analysis of a protein extract from spinach leaf showed a single band of 90.8 kDa. Soluble protein was purified to homogeneity from *S. frugiperda* cells infected with recombinant baculovirus harboring the isolated cDNA. The soluble protein had a molecular mass of 320 kDa, estimated by gel filtration chromatography, and a subunit size of 90.8 kDa. The purified protein had activity of both 6-phosphofructo-2-kinase (specific activity 10.4–15.9 nmol·min<sup>-1</sup>·mg protein<sup>-1</sup>) and fructose-2,6- bisphosphatase (specific activity 1.65–1.75 nmol·min<sup>-1</sup>·mg protein<sup>-1</sup>). The 6-phosphofructo-2-kinase activity was activated by inorganic phosphate, and inhibited by 3-carbon phosphorylated metabolites and pyrophosphate. In the presence of phosphate, 3-phosphoglycerate was a mixed inhibitor with respect to both fructose 6-phosphate and ATP. Fructose-2,6-bisphosphatase activity was sensitive to product inhibition; inhibition by inorganic phosphate was uncompetitive, whereas inhibition by fructose 6-phosphate was mixed. These kinetic properties support the view that the level of fructose 2,6-bisphosphate in leaves is determined by the relative concentrations of hexose phosphates, three-carbon phosphate esters and inorganic phosphate in the cytosol through reciprocal modulation of 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities of the bifunctional enzyme.

**Keywords:** fructose 2,6-bisphosphate, 6-phosphofructo-2-kinase, fructose-2,6-bisphosphatase, spinach leaf, *Spinacia oleracea*

**Abbreviations:** Fru-2,6- $P_2$ , fructose 2,6-bisphosphate; Fru-2,6- $P_2$ ase, fructose-2,6-bisphosphatase; Fru-6- $P$ , fructose 6-phosphate; 6PF2K, 6-phosphofructo-2-kinase; PFP, pyrophosphate:fructose 6-phosphate 1-phosphotransferase

**Enzymes:** 6-phosphofructo-2-kinase (EC 2.7.1.105); fructose-2,6-bisphosphatase (fructose-2,6-bisphosphate 2-phosphatase, EC 3.1.3.46).

Fructose 2,6-bisphosphate (Fru-2,6- $P_2$ ) is an important regulator of photosynthetic carbon metabolism in higher plants. It is a potent allosteric inhibitor of cytosolic fructose 1,6-bisphosphatase, which is responsible for the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate (Fru-6- $P$ ) during formation of sucrose from triose phosphates [1]. In leaves, Fru-2,6- $P_2$  contributes both to the coordination of sucrose synthesis with the rate of CO<sub>2</sub> fixation, and indirectly to the control of assimilate partitioning between sucrose and starch [1,2]. Direct evidence for the involvement of Fru-2,6- $P_2$  in the regulation of these processes is provided by studies of transgenic tobacco, kalanchoë, and arabidopsis in which changes in the rates of sucrose and starch synthesis correlated with changes in Fru-2,6- $P_2$  concentration when the latter was modified by genetic manipulation [3–6]. However, any explanation of how Fru-2,6- $P_2$  level serves to integrate photoassimilatory carbon partitioning must include a consideration of the factors that influence the concentration of this signal metabolite.

In common with other eukaryotes, the level of Fru-2,6- $P_2$  in higher plants is determined by the relative activities

of 6-phosphofructo-2-kinase (6PF2K) and fructose-2,6-bisphosphatase (Fru-2,6- $P_2$ ase), which catalyze its synthesis and degradation, respectively [7]. In leaves, both activities are subjected to reciprocal fine control by metabolic intermediates of the pathway of sucrose synthesis; 6PF2K activity is stimulated by Fru-6- $P$  and  $P_i$ , and inhibited by three-carbon phosphate esters (including 3-phosphoglycerate and dihydroxyacetone phosphate), whereas Fru-2,6- $P_2$ ase activity is inhibited by Fru-6- $P$  and  $P_i$  [1]. These properties allow the level of Fru-2,6- $P_2$  to respond sensitively to the availability of photosynthate and the accumulation of sucrose (the major photosynthetic end product), and provide the basis for a model describing the regulation of sucrose synthesis in leaves in the light [2].

Although both 6PF2K and Fru-2,6- $P_2$ ase activities have been measured in a range of plant tissues, detailed kinetic analyses are largely restricted to the activities from spinach leaves [1]. Consequently it is these activities that form the basis for our current understanding. However, the extent to which the reported properties of spinach 6PF2K and Fru-2,6- $P_2$ ase activities reflect those of the enzyme(s) *in vivo*

is uncertain. Much of the initial characterization of the activities was performed on relatively crude preparations of the enzyme(s) in which little effort was made to protect the sample from proteolysis during isolation [8–10]. There has been only one study in which a bifunctional enzyme has been purified to near-homogeneity [11]. That report identified two forms of the enzyme possessing both 6PF2K and Fru-2,6- $P_2$ ase activity. The smaller L-form of the enzyme (native  $M_r$  132,000) consisted of a variable group of catalytically active polypeptides with  $M_r$  of 44,000–70,000. Despite the presence of protease inhibitors, these polypeptides are likely to have been generated during extraction from the proteolytic degradation of a larger H-form (native  $M_r$  390,000, subunit  $M_r$  90,000) [11,12]. The affinity of the 6PF2K activity of the smaller L-form for its substrates and  $P_i$ , an allosteric activator, was lower than that of the corresponding activity of the larger H-form of the enzyme, whereas the corresponding affinity for its inhibitors was 10-fold greater [11]. Furthermore the ratio of 6PF2K activity to Fru-2,6- $P_2$ ase activity of the smaller form of the bifunctional enzyme was far lower than that of the larger form of the enzyme [11]. This is reminiscent of the enzyme from rat liver in which partial proteolysis destroyed 6PF2K activity while increasing Fru-2,6- $P_2$ ase activity [13]. Differences in the 6PF2K/Fru-2,6- $P_2$ ase ratio are a common feature of isoforms of the bifunctional enzyme from plants [11,12,14], suggesting that such proteolysis may be a widespread problem. The sensitivity of the plant bifunctional enzyme to degradation by endogenous proteases during isolation, and the demonstrable effects of proteolysis on the kinetic characteristics of the component activities of the enzyme compromise the evidence on which our current understanding of the regulation of photosynthetic carbon partitioning is based.

Additionally, a monofunctional Fru-2,6- $P_2$ ase has been purified from spinach leaves. This activity is specific for hydrolysis of Fru-2,6- $P_2$ , and is inhibited by Fru-6- $P$  and  $P_i$ , although the affinities for these inhibitors differ from those of the Fru-2,6- $P_2$ ase activity of the bifunctional enzyme. The protein has a native  $M_r$  of 50 000–76 000 with a subunit  $M_r$  of 33 000 [15]. The relationship between this monofunctional Fru-2,6- $P_2$ ase and the bifunctional enzyme is uncertain, and the role of the monofunctional enzyme in Fru-2,6- $P_2$ ase metabolism has yet to be resolved [15,16].

Recently cDNA clones encoding homologues of the mammalian bifunctional enzyme have been isolated from potato leaf [17] and arabidopsis hypocotyls [18]. The deduced amino-acid sequence of both clones contain a region in which about 40–50% of the residues are identical to those of the 400-residue “catalytic core” of the mammalian, avian and yeast enzymes [19]. When expressed in *E. coli*, the proteins encoded by the two plant cDNA display both 6PF2K and Fru-2,6- $P_2$ ase activities [17,18]. These developments provide the opportunity to examine the kinetic properties of plant 6PF2K/Fru-2,6- $P_2$ ase purified from a heterologous expression system, thus circumventing problems associated with potential modification of the enzyme by endogenous plant proteases during extraction. Here we report on the kinetic properties of a spinach bifunctional 6PF2K/Fru-2,6- $P_2$ ase produced in insect cells using a baculovirus expression system.

## Experimental Procedures

### Materials

Superscript Choice System for cDNA synthesis, TC100 medium, SF-900 II serum-free medium, fetal bovine serum and FastBac expression system were from Invitrogen Life Technologies (Paisley, UK). Genescreen Plus membrane and [ $\alpha$ - $^{32}$ P]dCTP were from NEN Life Science Products (Hounslow, Middlesex, UK), and restriction enzymes were from New England Biolabs (Hitchin, Herts, UK). Chromatography media and columns were from Amersham Biosciences (Little Chalfont, Bucks, UK). Pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) was purified from mature tubers of potato (*Solanum tuberosum*), as described previously [20]. Other coupling enzymes and Triton X-100 were supplied by Roche Diagnostics (Lewes, East Sussex, UK). Phenol was from Qbiogene (Harefield, Middlesex, UK) and all other chemicals were from Sigma-Aldrich or Merck (both of Poole, Dorset, UK).

### CDNA library construction

Total RNA was isolated from recently expanded mature leaves of *Spinacia oleracea*, as described previously [21]. PolyA<sup>+</sup> RNA was purified using the Oligotex purification system (Qiagen, Crawley, West Sussex, UK), and 3  $\mu$ g was used for cDNA synthesis using oligo dT<sub>(n)</sub> primers. Size selected cDNA (>1 kbp) was cloned into EcoRI-digested lambda ZAP II (Stratagene, Amsterdam, the Netherlands). The host bacterial strain was XL1-Blue (Stratagene).

### Northern analysis

Approximately 20  $\mu$ g total RNA were separated in 1.4% agarose gels containing 6.3% formaldehyde and transferred by capillary action to Hybond-N membrane (Amersham Biosciences).

### Southern analysis

Genomic DNA was isolated from mature spinach leaves by the CTAB extraction procedure [22]. DNA was digested with restriction enzymes (10 U· $\mu$ g<sup>-1</sup> DNA) in buffer supplied by the manufacturer for 24 h. The DNA fragments were separated on a 0.8% agarose gel and transferred to Hybond-N membrane by capillary transfer.

### Probe labeling and hybridization

DNA probes for both Southern and Northern analysis were labeled with [ $\alpha$ - $^{32}$ P]dCTP using Ready-to-Go labeling reactions and separated from unincorporated nucleotides through ProbeQuant G-50 Micro-columns (Amersham Biosciences). The complete cDNA sequence was used as template for probe synthesis. Membranes were hybridized in ExpressHyb hybridization solution (Clontech, Basingstoke, Hampshire, UK), according to the manufacturer's instructions. Following hybridization with the probe, membranes were rinsed in 2 × NaCl/Cit/0.5% SDS at room temperature and then washed twice in 0.2 × NaCl/Cit/0.1% SDS at 42 °C, each time for 30 min.

### Sequencing and sequence analysis

DNA sequences were determined by cycle sequencing using an ABI Prism automated sequencer (Applied Biosystems Inc., Warrington, Cheshire, UK) at the Durham University Sequencing Service and Department of Pathology, University of Oxford, UK. Sequence data were processed using DNASTRIDER and GCG computer programs.

### Preparation of antibodies

The coding region from the 6PF2K/Fru-2,6- $P_2$ ase cDNA was amplified from the lambda ZAP II-derived clone by PCR using the primer 5'-TTAGGAGAGAGACAT ATGGG-3' and the M13 reverse primer. The amplified fragment was cloned in-frame into pET 30 expression vector (Invitrogen Life Technologies) using *Nde*I and *Nof*I restriction sites and transformed into *E. coli* strain BL21(ΔDE3). Protein expression was induced in cells growing logarithmically in terrific broth [23] at 37 °C by adding isopropyl thio-β-D-galactoside at a final concentration of 1 mM. Bacteria were harvested, lysed and the inclusion bodies were isolated by centrifugation [23].

Approximately 75 mg of insoluble protein derived from inclusion bodies were fractionated by continuous-elution SDS/PAGE on a 35 × 100 mm 7% acrylamide gel using a Model 491 Prep Cell (Bio-Rad, Hemel Hempstead, Herts, UK), according to the manufacturer's instructions. Fractions containing the pure recombinant protein ( $M_r \approx 90,800$ ) were identified by analytical SDS/PAGE and the protein recovered from the pooled fractions by methanol/chloroform precipitation. The protein was redissolved in 1 mL 6 M guanidinium/HCl and dialysed exhaustively against NaCl/ $P_i$ . The resulting protein suspension was used to raise polyclonal antibodies in New Zealand white rabbits at Harlan Sera Laboratories (Loughborough, Leics, UK).

### PAGE and immunoblotting

Analytical SDS/PAGE was performed using a Phastgel system (Amersham Biosciences) run according to the manufacturer's recommended conditions. For immunochemical analysis, protein was transferred onto a poly(vinylidene difluoride) membrane (Millipore, Watford, Herts, UK) and probed with rabbit anti-(6PF2K/Fru-2,6- $P_2$ ase) Ig at a 1 : 1000 dilution. Primary antibodies bound to the membrane were detected using alkaline phosphatase-conjugated secondary goat anti-(rabbit IgG) Ig, as described previously [24].

### Expression in *Spodoptera frugiperda* cells

Routine subcultures of *S. frugiperda* (cell line SF21) were grown in TC100 medium supplemented with 10% fetal bovine serum and 0.1% Pluronic F-68 in shake flasks at 80 r.p.m. and 27 °C. Recombinant baculovirus was engineered using the FastBac system from Invitrogen Life Technologies, according to the manufacturer's instructions. The primers 5'-TTAGGATCCAGAAAAATGGGG-3' and 5'-AA-CAAACAGCGCGCGGGCACTTTAATCC-3' were used in PCR to amplify the coding region of the cDNA and introduce appropriate restriction sites. The plasmid pFAST-Bac-1 and the PCR product were ligated after digestion with

*Bam*HI and *Nof*I. The subsequent plasmid was used to produce recombinant baculovirus particles. Large-scale cultures of baculovirus (666 mL) were grown in a 2-L flask in a mixture comprising 75% SF-900 II and 25% TC100/10% fetal bovine serum/0.1% F-68. Amplification of viral stocks was carried out using a multiplicity of infection of  $\leq 0.1$  for at least 4 days. For protein production, 666 mL of cells were inoculated with recombinant baculovirus at a multiplicity of infection of 2–3 and grown for 60–72 h.

### Purification of recombinant 6PF2K/Fru-2,6- $P_2$ ase

*S. frugiperda* cells were harvested from  $\approx 700$  mL of cell culture by centrifugation at 1000 g for 10 min. The cells were resuspended in 100 mL of buffer A (50 mM Tris/acetate (pH 7.8), 5 mM Mg/acetate, 2.5 mM dithiothreitol, 1  $\mu$ g·mL<sup>-1</sup> leupeptin) supplemented with 100 mM K/acetate (pH 7.8), 0.1 mg·mL<sup>-1</sup> 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1  $\mu$ g·mL<sup>-1</sup> E-64 and 1  $\mu$ g·mL<sup>-1</sup> pepstatin and lysed by sonication until >95% of the cells were broken. Insoluble material was removed by centrifugation at 10 000 g for 20 min. The supernatant was adjusted to 3% poly(ethylene glycol) 4000 by adding 0.11 vol. of a 30% poly(ethylene glycol) solution in buffer A. After 5 min, precipitated protein was removed by centrifugation at 10,000 g for 20 min. The supernatant was adjusted to 15% poly(ethylene glycol) by the addition of 0.67 vol. of 30% poly(ethylene glycol) in buffer A, and after 10 min centrifuged at 10,000 g for 20 min. The resulting pellet was resuspended in 50 mL of buffer A containing 50 mM KCl and applied to a 50-mL DEAE-Sepharose column equilibrated in the same buffer. Protein was eluted with a 450-mL linear gradient of 50–500 mM KCl in buffer A. Fractions containing the peak of 6PF2K activity were combined and applied to a 20-mL Blue Sepharose FF column equilibrated in buffer A. After loading, the Blue Sepharose column was washed with 20 mL of buffer A containing 14 mM ATP and 28 mM Mg/acetate. Protein was eluted from the column with 200 mL buffer A containing 9 mM ATP, 18 mM Mg/acetate, 2 mM Fru-6- $P$ , 2.5 mM glycerol 3-phosphate, 2.5 mM phosphoenolpyruvate and 200 mM K/acetate (pH 7.8). The active fractions were combined and concentrated by ultrafiltration (YM10 membrane, Millipore) to a final volume of 10 mL. This was diluted to 50 mL with buffer B [25 mM Tris/acetate (pH 7.8), 5 mM Mg/acetate, 5 mM dithiothreitol and concentrated again to 10 mL]. The concentrated sample was applied to a Mono-Q HR5/5 column equilibrated with buffer B and eluted with a linear gradient over 20 mL of 0–500 mM KCl. The eluate was collected in 0.5-mL aliquots. Fractions from the Mono-Q column were purified further by gel filtration chromatography by applying 200-μL samples to a Superose 12 HR10/30 column equilibrated with buffer B supplemented with 150 mM NaCl. Samples were eluted at a flow rate of 0.3 mL·min<sup>-1</sup> and collected in 200-μL fractions.

### Enzyme assays

The activities of 6PF2K and Fru-2,6- $P_2$ ase were determined by measuring the formation or disappearance of Fru-2,6- $P_2$  [25]. Unless otherwise specified, 6PF2K activity was assayed in 100 mM Tris/Cl (pH 7.8), 4 mM MgCl<sub>2</sub>,



2 mM ATP, 2 mM Fru-6-P, 5 mM  $\text{KH}_2\text{PO}_4$ , 5 mM dithiothreitol, 2  $\text{mg}\cdot\text{mL}^{-1}$  BSA and 20 mM KF, in a final volume of 200  $\mu\text{L}$ . The assay for Fru-2,6- $P_2$ ase activity normally contained 50 mM K/Hepes (pH 7.5), 5 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol, 2  $\text{mg}\cdot\text{mL}^{-1}$  BSA and 100 nM Fru-2,6- $P_2$ . In both assays, activity was calculated by measuring the amount of Fru-2,6- $P_2$  present in 10- $\mu\text{L}$  aliquots (usually four) of the reaction mixture removed at timed intervals after the beginning of the assay. Each aliquot was added to 40  $\mu\text{L}$  250 mM KOH immediately after withdrawal from the reaction mixture to inactivate the enzymes, and the Fru-2,6- $P_2$  content of a 10- $\mu\text{L}$  sample of the resulting mixture was determined by measuring its ability to activate PFP. For each determination of 6PF2K and Fru-2,6- $P_2$ ase activity, the activation of PFP was calibrated against an internal standard of authentic Fru-2,6- $P_2$  added to an aliquot of the assay mixture that had been removed at the beginning of the assay and acid-treated (to remove endogenous Fru-2,6- $P_2$ ) prior to analysis. The activity of PFP was measured spectrophotometrically using an automated microplate reader (model EL340; Bio-Tek Instruments, Winooski, Vermont, USA) in a final volume of 200  $\mu\text{L}$ , by coupling the production of fructose 1,6-bisphosphate to the oxidation of NADH as described previously [26]. The concentration of Fru-2,6- $P_2$  used as an internal standard was determined enzymatically after hydrolysis of an aliquot of the concentrated stock solution to Fru-6-P [25]. For kinetic studies, contaminating  $P_i$  was removed from Fru-6-P and ATP [27]. One unit of enzyme activity (U) is the amount of enzyme that synthesizes or degrades 1  $\mu\text{mol}$  of Fru-2,6- $P_2$  per minute at 25 °C.

#### Determination of kinetic parameters

All kinetic constants and corresponding asymptotic standard errors were determined by nonlinear regression analysis of the untransformed data using the Marquardt-Levenberg algorithm [28]. Data were fitted to the appropriate kinetic equations using SIGMAPLOT 2000 (SPSS, Chicago, Illinois, USA). In each analysis the correlation coefficient was greater than 0.975. Kinetic constants are those defined by Cornish-Bowden [29].

#### Protein determination

Protein concentrations were determined by the Bradford method [30] using bovine  $\gamma$ -globulin as a standard.

## Results

#### Isolation of cDNA for spinach leaf 6PF2K/Fru-2,6- $P_2$ ase

A  $\lambda$  phage cDNA library constructed from mature spinach leaves was screened with a 450-bp EST clone from *Pinus taeda* (partial sequence, GenBank accession number H75207) homologous to the Fru-2,6- $P_2$ ase domain of the bifunctional enzyme from mammalian sources. From  $\sim 3 \times 10^5$  unamplified plaques, two strongly hybridizing cDNA clones were isolated. The larger clone (GenBank accession number AF041848) contained 2520 bp (excluding the polyA<sup>+</sup> tail) and possessed a single ORF beginning at nucleotide 29 and terminating with a 242-bp 3' noncoding region. This sequence encodes a polypeptide of 750 amino acids with a predicted

molecular mass of 83,374 Da and a theoretical pI of 5.88. The DNA sequence of the second clone, which was inserted into the vector in the opposite orientation, was 16 bp shorter at the 5' end but otherwise identical to that of the larger clone.

Alignment of the deduced amino-acid sequence against 6PF2K/Fru-2,6- $P_2$ ase from other sources (Figure 1) revealed two distinct regions of similarity. The section of the polypeptide from about Ile351 to the C-terminus was very similar to the known sequences of 6PF2K/Fru-2,6- $P_2$ ase from other plants (potato tuber, 88%; arabidopsis hypocotyl, 88%; mangrove, 87%; maize leaf, 81%) and similar to those from other eukaryotes (mammalian liver, skeletal muscle, brain and testis, 45–47%). This region contains the domains for both 6PF2K and Fru-2,6- $P_2$ ase activities and forms the catalytic core of the bifunctional enzyme [19]. Within this region all nine residues known to be crucial for Fru-2,6- $P_2$ ase activities in the liver isoform of the mammalian enzyme are conserved in the same relative positions within the spinach leaf sequence (Figure 1). Similarly, 17 of the 21 residues identified as being important for 6PF2K activity in the rat liver or testes isozymes are conserved in the alignment of the spinach leaf enzyme (Figure 1). The N-terminal region from Met1 to Ala350 is similar to the N-terminal region of corresponding 6PF2K/Fru-2,6- $P_2$ ase cDNA from arabidopsis (56% identity) and mangrove (59% identity), and to a partial cDNA from potato (58% identity), but is unrelated to sequences of 6PF2K/Fru-2,6- $P_2$ ase from nonplant sources.

#### Detection of the gene, transcript and protein for 6PF2K/Fru-2,6- $P_2$ ase in spinach

A probe generated from the cDNA hybridized to multiple fragments on blots of genomic DNA digested with *Bam*HI, *Eco*RI or *Hin*DIII, confirming the presence of this sequence within the spinach genome (data not shown). On blots of total RNA from spinach leaves, the same probe hybridized to a single band of  $\sim 2500$  bp, corresponding to the length of the isolated cDNA (Figure 2A).

Expression of the coding region of 6PF2K/Fru-2,6- $P_2$ ase in *E. coli* led to the production of large amounts of insoluble protein. Antibodies were raised against the recombinant polypeptide purified from inclusion bodies. These antibodies detected a single band with an apparent molecular mass of 90.8 kDa on immunoblots of spinach leaf protein (Figure 2B). Although both 6PF2K and Fru-2,6- $P_2$ ase activities were detectable in extracts of *E. coli* expressing the recombinant protein, the kinetic properties of the enzyme from this source were not studied in detail because the majority of the soluble activity was associated with several truncated proteins from which the full-length 90.8 kDa polypeptide could not be separated by conventional non-denaturing chromatographic techniques (data not shown).

#### Expression and purification of soluble 6PF2K/Fru-2,6- $P_2$ ase

Soluble, recombinant 6PF2K/Fru-2,6- $P_2$ ase was produced by expression in *S. frugiperda* cell culture using a baculovirus expression system. The recombinant enzyme was purified to apparent homogeneity by poly(ethylene glycol) precipitation, followed by chromatography on DEAE-

Spinach	MGAGTSKNSD	STSHGDGDGE	ERDEYEGGGQ	LYVSLKMENY	TLNADLLPHV	FGSASIVGWS
Arabidopsis	MGSGASKNTE	E....DDGGS	N....GGGGQ	LYVSLKMENS	KVEGELTPHV	YGSPLIGSW
Spinach	DSSRALPLER	ESTSMWQLSF	VVPPNHETLD	FKFLLRPKDS	NNPCIIEEGP	NRIISGGRLO
Arabidopsis	DPSKALEMOR	ESALMSLSLF	VVPPDHETLD	FKFLLKPKNR	NTFCIVEEGE	NRLITGGSLO
Spinach	GDARAALFRL	NADAVLEYRV	CINADRISFF	DLASSWRAYQ	ENIHPSTVRG	ILDVSMNSET
Arabidopsis	GDARLALFRL	EGDVIVEFRV	FINADRVSEI	DLATSWRAYR	ENLQPSTVRG	IPDVSINPDF
Spinach	CESSSSANL	ELDLERYVVP	SPATSATSGL	VYPANLAETP	RYISARKYSS	ESDFSKSGDT
Arabidopsis	KSAECPLESL	ELDLAHYEVP	APAPSANSYL	VYAADNAENP	RSLSASGSFR	NDSTPKAAQR
Spinach	GSQPIGPTAD	....KEMEV	KIQDLAKVST	TSAAVEDKLA	TTASPTQKQE	SHK.GVVFDR
Arabidopsis	NESDSGVTVD	GSPSAKEMTI	VVPSSNIYS	AFGEAESKSV	ETLSPVSARK	MVRKDSFVDR
Spinach	GVGSPLRVKS	TSAGTEADDV	KLGSNGKTAM	PAAAGAVAAA	AVADQMLGPK	EDRHIAIVLV
Arabidopsis	GVGSPLRVKS	LSASSELIOT	K...QIKNSM	PAAAGAVAAA	AVADQMLGPK	EDRHIAIVLV
Rat liver		MSREMELT	QTRLQKIWIP	HSSSSSVLQR	RRG.SSIPQF	TNSPTVMIMV
Spinach	GLPARGKTFT	AAKLTRYLRW	LGHDTKHENV	GKYRRLKLG.	TNQSAEFFRG	DNTEGIEARN
Arabidopsis	GLPARGKTFT	AAKLTRYLRW	LGHDTKHENV	GKYRRLKHG.	VNMSADFFRA	DNPEGVEART
Rat liver	GLPARGKTYI	STKLTRYLNV	IGTPTKVENL	GOYRREAVSY	..RNYEFFRP	DNTEAQLISK
Spinach	EVAALAMEDM	ISWM.QEGGQ	VGIFDATNST	RERRNMLMKL	A.EGNCKIIF	LETLCYDERI
Arabidopsis	EVAALAMEDM	IAMW.QEGGQ	VGIFDATNST	RVRNMLMMK	A.EGCKKIIF	LETLCNDERI
Rat liver	QCALAALKDV	HKYLSREEGH	VAVEDATNTT	RERRSLILQF	AKEHGKVF	IESICNDPEI
Spinach	IERNIRLWQ	QSPDYAEQPD	FEAGYDFPR	RLDNYEKVYE	PVEEG....	SYIKMIDTVS
Arabidopsis	IERNIRLWQ	QSPDYSEEMD	FEAGVDFPRD	RLANYEKVYE	PVEEG....	SYIKMIDMVS
Rat liver	IAENIKQVRL	GSEDY.IDCD	QEKVLEDFLK	RIECYINYO	ELDEELDSHL	SYIKIFDVGT
Spinach	GNGGQIQVNN	ISGYLPGRHV	FFLVNTHLTP	RPILLARHCE	SQDNVFRARIG	GDSLSLDTGE
Arabidopsis	GNGGQIQVNN	ISGYLPGRHV	FFLVNTHLTP	RPILLARHCE	SMDNVGRIGRIG	GDSVISDSGK
Rat liver	...RYMVNR	VQDHVQSSTA	YYLMNIHVTF	RSYILCRHGE	SELNLRGRIG	GDSGLSARGK
Spinach	LYAKKLANFV	EKRLKPERAA	SIWTSTLHRS	ISTANHIVGF	PKVQWRALDE	INAGACDGMT
Arabidopsis	LYAKKLASFV	EKRLKSEKAA	SIWTSTLQRT	NLTASSIVGF	PKVQWRALAE	INAGVCDGMT
Rat liver	QYAYALANFI	..RSQGISSL	KVWTSHMKRT	IQTAEL.GV	EYEQWKALME	IDAGVCEEMT
Spinach	YLEIKMDMPE	EYESREKDKL	RYRYPGRGESY	LDVIQRLPEV	IIELERQRAP	VVVISHOAVL
Arabidopsis	YEEVKKNMPE	EYESRKDKL	RYRYPGRGESY	LDVIQRLPEV	IIELERQRAP	VVVISHOAVL
Rat liver	YEETQEHYPE	EFALRDQDKY	RYRYPKGESY	EDLVQRLPEV	IMELERQE.N	VLVICHQAVM
Spinach	RALYAYFADR	PLKEIPHIEV	PLDTIIEIQM	GVTGVQEKRY	KLMD	
Arabidopsis	RALYAYFADR	PLKEIPQIEM	PLDTIIEIQM	GVSGVQEKRY	KLMD	
Rat liver	RCLLAYFLDK	SSDELEYLKC	PLDTVLKLT	VAYGCRVESI	YLNVEAVNTH	RDKPENVDIT
Rat liver	REAEELDTV	PAHY				

Figure 1. Alignment of the amino-acid sequences of 6PF2K/Fru-2,6-P<sub>2</sub>ase from various sources. The origin of the sequences compared are spinach (GenBank accession number AF041848), arabidopsis (AF190739) and rat (liver isozyme, Y00702). Grey boxes show identity between the spinach and other sequences. Residues highlighted in black are those previously identified as essential for 6PF2K or Fru-2,6-P<sub>2</sub>ase. Ile-135, referred to in the text, is indicated (▼).

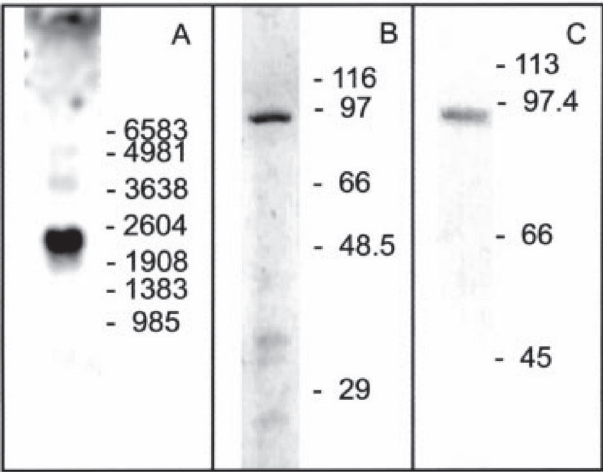
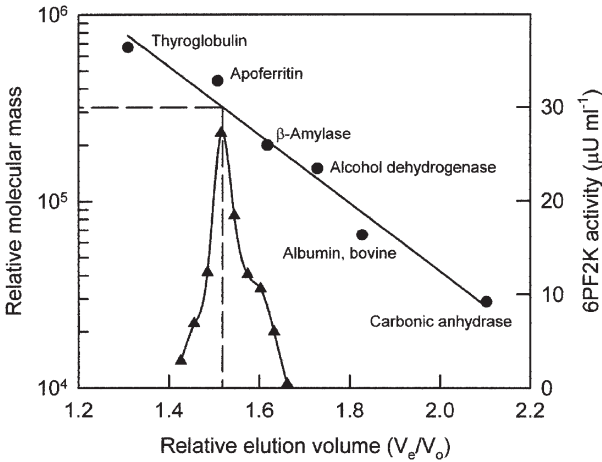


Figure 2. Detection of 6PF2K/Fru-2,6-P<sub>2</sub>ase transcript and protein in spinach. (A) Northern blot of total RNA from spinach leaves (B) Western blot of total protein extract of spinach leaves, and (C) SDS/PAGE of 1 µg of recombinant protein purified from *S. frugiperda* stained with Coomassie blue. Values alongside each track indicate the size of molecular mass standards presented as (A) nucleotides, and (B,C) kDa.

Sephacrose, Blue-Sepharose, Mono Q and Superose-12. The yield of enzyme based on 6PF2K activity was typically 10%. The purified protein eluted with an apparent molecular mass of 320 kDa during gel filtration (Figure 3) and yielded a single polypeptide with a molecular mass of 90.8 kDa when analyzed by SDS/PAGE (Figure 2C).

Kinetic properties of recombinant 6PF2K/Fru-2,6-P<sub>2</sub>ase

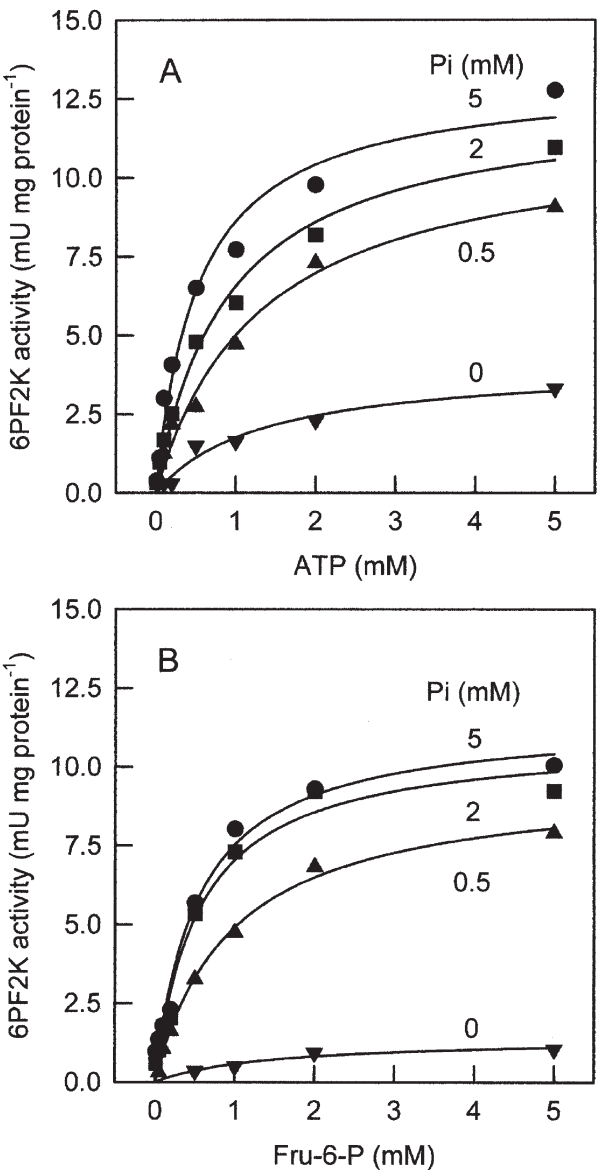
The purified recombinant protein possessed both 6PF2K and Fru-2,6-P<sub>2</sub>ase activities. The 6PF2K activity was markedly stimulated by P<sub>i</sub>. This activity displayed standard Michaelis-Menten kinetics with respect to both ATP and Fru-6-P in the presence and absence of P<sub>i</sub> (Figure 4). Activation by P<sub>i</sub> resulted from both an increase in V<sup>app</sup><sub>max</sub> and a decrease in K<sup>app</sup><sub>m</sub> for each substrate (Table 1). This activity was also inhibited by a range of three-carbon phosphate esters and by PP<sub>i</sub>. Each of these compounds displayed hyperbolic inhibition kinetics at fixed concentrations of ATP and Fru-6-P. In the presence of 2 mM P<sub>i</sub>, 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate were all effective inhibitors at micromolar concentrations (Table 2). The enzyme activity was less sensitive to inorganic pyrophosphate,



**Figure 3. Native molecular mass of recombinant 6PF2K/Fru-2,6- $P_2$ ase.** Elution of 6PF2K activity from a Superose-12 gel filtration column ( $\blacktriangle$ ). The elution of other proteins used to calibrate the column are as indicated ( $\bullet$ ). Elution volume ( $V_e$ ) is expressed relative to the void volume of the column ( $V_0$ ) determined from the elution of blue dextran.

glycerol 3-phosphate and dihydroxyacetone phosphate under the conditions used in this investigation (Table 2). We chose to study inhibition by 3-phosphoglycerate in more detail by examining the effect of this compound on the kinetic response of 6PF2K activity to varying substrate concentrations. The activity displayed normal hyperbolic kinetics over the range 0–1.0 mM 3-phosphoglycerate (Figure 5). Inhibition was caused by progressive decreases in  $V_{max}^{app}$  and increases in  $K_m^{app}$  for both ATP and Fru-6-P as the concentration of 3-phosphoglycerate was increased (Table 3). Inhibition by 3-phosphoglycerate was overcome by increasing concentrations of  $P_i$ , which increased  $V_{max}^{app}$  and decreased  $K_m^{app}$ . In the presence of 2 mM Fru-6-P, 0.2 mM 3-phosphoglycerate and 2 mM  $P_i$ ,  $V_{max}^{app}$  was  $7.00 \pm 0.38$  mU·mg protein $^{-1}$  and  $K_m^{app}$  for ATP was  $0.46 \pm 0.08$  mM; the corresponding values in the presence of 10 mM  $P_i$  were  $11.11 \pm 0.42$  mU·mg protein $^{-1}$  and  $0.34 \pm 0.05$  mM, respectively (Figure 6). Similar effects were observed when Fru-6-P was the varied substrate (data not shown).

As Fru-2,6- $P_2$ ase from plants is reported to be sensitive to product inhibition [1], we determined the effect of both Fru-6-P and  $P_i$  on the Fru-2,6- $P_2$ ase activity associated with the recombinant bifunctional enzyme. The activity of Fru-2,6- $P_2$ ase displayed normal hyperbolic substrate kinetics at each of the concentrations of  $P_i$  and Fru-6-P studied (Figure 7). Over the range 0–5.0 mM,  $P_i$  was an uncompetitive inhibitor. Nonlinear



**Figure 4. Effect of  $P_i$  on the affinity of 6PF2K for Fru-6-P and ATP.** Enzyme activity was measured over the range 0.01–5.0 mM ATP at 2 mM Fru-6-P (A), and 0.01–5.0 mM Fru-6-P at 2 mM ATP (B). The concentration of  $P_i$  was 0 mM ( $\nabla$ ), 0.5 mM ( $\blacktriangle$ ), 2.0 mM ( $\blacksquare$ ), or 5.0 mM ( $\bullet$ ). Each value is a single determination of activity based on a 4-point timecourse of Fru-2,6- $P_2$  production. Hill coefficients were between  $0.82 \pm 0.18$  and  $0.90 \pm 0.09$  with respect to ATP (A) and between  $1.05 \pm 0.09$  and  $1.15 \pm 0.19$  with respect to Fru-6-P (B); none of these values was significantly different from unity.

**Table 1. Effect of  $P_i$  on the kinetic constants of 6PF2K.** Enzyme activity was measured at the concentration of ATP or Fru-6-P shown in Figure 4 while the concentration of the cosubstrate was maintained at 2 mM. Kinetic constants were obtained by fitting data to the equation for a single-substrate Michaelis–Menten reaction and are expressed as the best-fit estimate  $\pm$  SE from eight measurements.

$P_i$ (mM)	ATP		Fru-6-P	
	$V_{max}^{app}$ (mU·mg protein $^{-1}$ )	$K_m^{app}$ (mM)	$V_{max}^{app}$ (mU·mg protein $^{-1}$ )	$K_m^{app}$ (mM)
0	$4.08 \pm 0.49$	$1.32 \pm 0.40$	$1.41 \pm 0.18$	$1.41 \pm 0.47$
0.5	$11.47 \pm 0.99$	$1.29 \pm 0.28$	$9.58 \pm 0.33$	$0.92 \pm 0.09$
2.0	$12.45 \pm 0.62$	$0.90 \pm 0.13$	$10.92 \pm 0.61$	$0.55 \pm 0.10$
5.0	$13.16 \pm 0.82$	$0.53 \pm 0.11$	$11.51 \pm 0.60$	$0.53 \pm 0.09$



**Table 2. Inhibition of 6-phosphofructo-2-kinase activity by phosphate esters.** Enzyme activity was determined using 2 mM Fru-6-P, 2 mM ATP. The concentration of phosphate ester producing half-maximum inhibition ( $I_{0.5}$ ) is presented as the best-fit estimate  $\pm$  SE from eight measurements.

Compound	$I_{0.5}$ (mM)
Pyrophosphate	$0.106 \pm 0.018$
Glycerol 3-phosphate	$8.07 \pm 0.305$
Phosphoenolpyruvate	$0.045 \pm 0.007$
2-Phosphoglycerate	$0.029 \pm 0.004$
3-Phosphoglycerate	$0.084 \pm 0.005$
Dihydroxyacetone phosphate	$0.737 \pm 0.218$

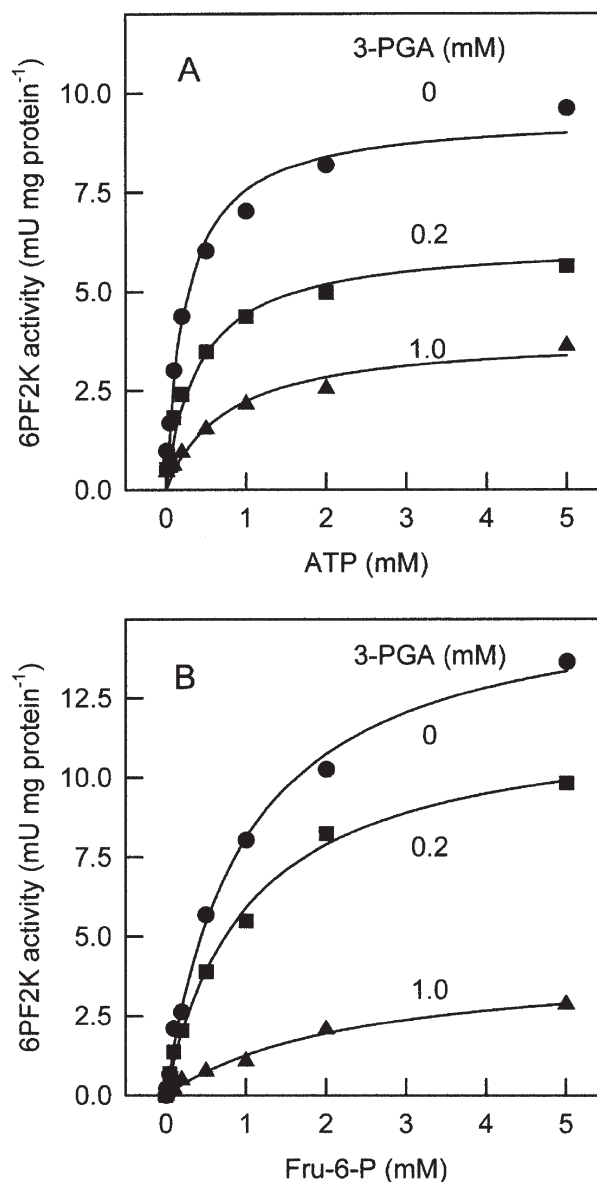
regression analysis of the untransformed data yielded the following values:  $V_{\max}$ ,  $1.75 \pm 0.12$  mU·mg protein<sup>-1</sup>;  $K_m$ ,  $65.9 \pm 4.58$  nM;  $K_{iu}$ ,  $1.20 \pm 0.11$  mM, in which the values are the best-fit estimates  $\pm$  SE from 21 measurements. Attempts to fit the same data to the kinetic equation describing mixed inhibition produced an estimate for  $K_{ic} > 100$  mM, demonstrating that there was a negligible competitive component to the inhibition of Fru-2,6-P<sub>2</sub>ase activity by P<sub>i</sub>. In contrast, comparable analysis of the effects of 0–1.0 mM Fru-6-P yielded the following constants:  $V_{\max}$ ,  $1.65 \pm 0.22$  mU·mg protein<sup>-1</sup>;  $K_m$ ,  $61.9 \pm 3.17$  nM;  $K_{ic}$ ,  $0.65 \pm 0.03$  mM;  $K_{iu}$ ,  $1.55 \pm 0.14$  mM. These values indicate that Fru-6-P is a mixed inhibitor with significant competitive and uncompetitive components.

Based on the  $V_{\max}$  values for the two activities obtained in these analyses, the 6PF2K/Fru-2,6-P<sub>2</sub>ase ratio of the recombinant bifunctional spinach enzyme was 6.5–9.6.

## Discussion

The recombinant protein investigated in the present study is likely to represent the complete bifunctional 6PF2K/Fru-2,6-P<sub>2</sub>ase from spinach leaves. The length of the isolated cDNA corresponds closely to the size of the transcript identified by hybridization against spinach leaf RNA. Moreover, the protein expressed in insect cells is the same size as the polypeptide identified in crude extracts of spinach leaves by antibodies raised against the recombinant protein. The size of this protein is very similar to that of the H-form of the bifunctional enzyme previously purified from spinach leaves [11]. More recently, transcripts and polypeptides of similar sizes have been identified in arabidopsis seedlings [18].

The structure of the spinach leaf enzyme studied in this paper conforms to the pattern of all other bifunctional 6PF2K/Fru-2,6-P<sub>2</sub>ase proteins so far studied [7]. It is composed of four regions; a central core consisting of the 6PF2K and Fru-2,6-P<sub>2</sub>ase domains that are flanked by variable N- and C-termini. As might be anticipated, the central catalytic core shares a high degree of sequence identity with the corresponding region of the bifunctional enzyme from other eukaryotic sources (Figure 1). Notably, only four of the known catalytic residues are not conserved in the same relative positions in the spinach and mammalian enzyme. However, one of these (Lys479, spinach) is found in an adjacent position in the strict alignment (Figure 1). Furthermore, for each of the other three discrepancies, the amino-acid substitutions



**Figure 5. Effect of 3-phosphoglycerate on the affinity of 6PF2K for Fru-6-P and ATP.** Enzyme activity was measured over the range 0.01–5.0 mM ATP at 2 mM Fru-6-P (A), and 0.01–5.0 mM Fru-6-P at 2 mM ATP (B) in the presence of 2 mM P<sub>i</sub>. The concentration of 3-phosphoglycerate was 0 mM (●), 0.2 mM (■), or 1.0 mM (▲). Each value is a single determination of activity based on a four-point timecourse of Fru-2,6-P<sub>2</sub> production. Hill coefficients were between  $0.89 \pm 0.11$  and  $1.26 \pm 0.20$  with respect to ATP (A) and between  $0.87 \pm 0.14$  and  $0.92 \pm 0.08$  with respect to Fru-6-P (B); none of these values was significantly different from unity. (3-PGA = 3-phosphoglycerate)

found in the spinach sequence (Ser441, Gln531, Asn536) are also present in the bifunctional enzymes from arabidopsis [18], potato [17], mangrove (AB061797) and maize (AF007582).

A striking feature of the deduced amino-acid sequence of spinach 6PF2K/Fru-2,6-P<sub>2</sub>ase is the size of the N-terminal region preceding the catalytic core. This 350-residue section contains several motifs that are found in the corresponding region of the bifunctional enzyme from other plants, but



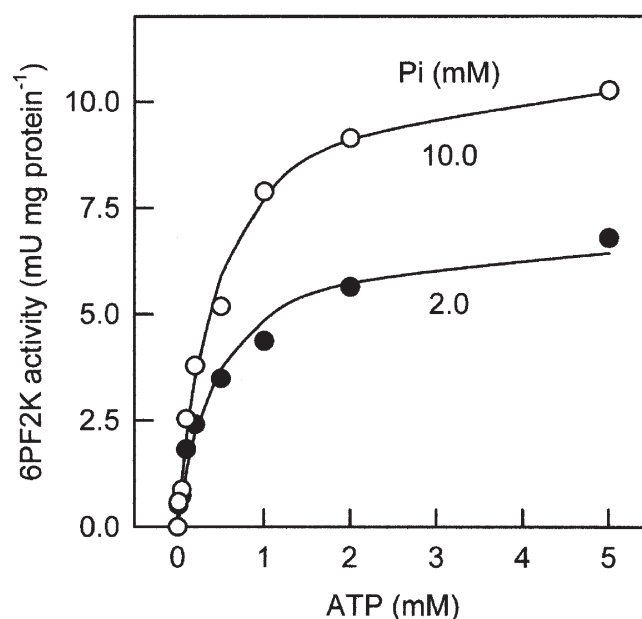
**Table 3. Effect of 3-phosphoglycerate on the kinetic constants of 6PF2K.** Enzyme activity was measured in the presence of 2 mM  $P_i$ . The concentration of either ATP or Fru-6-P was varied as shown in Figure 5 while the concentration of the cosubstrate was maintained at 2 mM. Kinetic constants were obtained by fitting data to the equation for a single-substrate Michaelis-Menten reaction and are expressed as the best-fit estimate  $\pm$  SE from eight measurements.

3-Phosphoglycerate (mM)	ATP		Fru-6-P	
	$V_{\max}^{\text{app}}$ (mU·mg protein $^{-1}$ )	$K_m^{\text{app}}$ (mM)	$V_{\max}^{\text{app}}$ (mU·mg protein $^{-1}$ )	$K_m^{\text{app}}$ (mM)
0	10.40 $\pm$ 0.75	0.32 $\pm$ 0.09	15.92 $\pm$ 0.54	0.96 $\pm$ 0.09
0.2	6.25 $\pm$ 0.73	0.41 $\pm$ 0.16	11.93 $\pm$ 0.41	1.02 $\pm$ 0.09
1.0	3.89 $\pm$ 0.38	0.74 $\pm$ 0.13	4.25 $\pm$ 0.34	2.36 $\pm$ 0.40

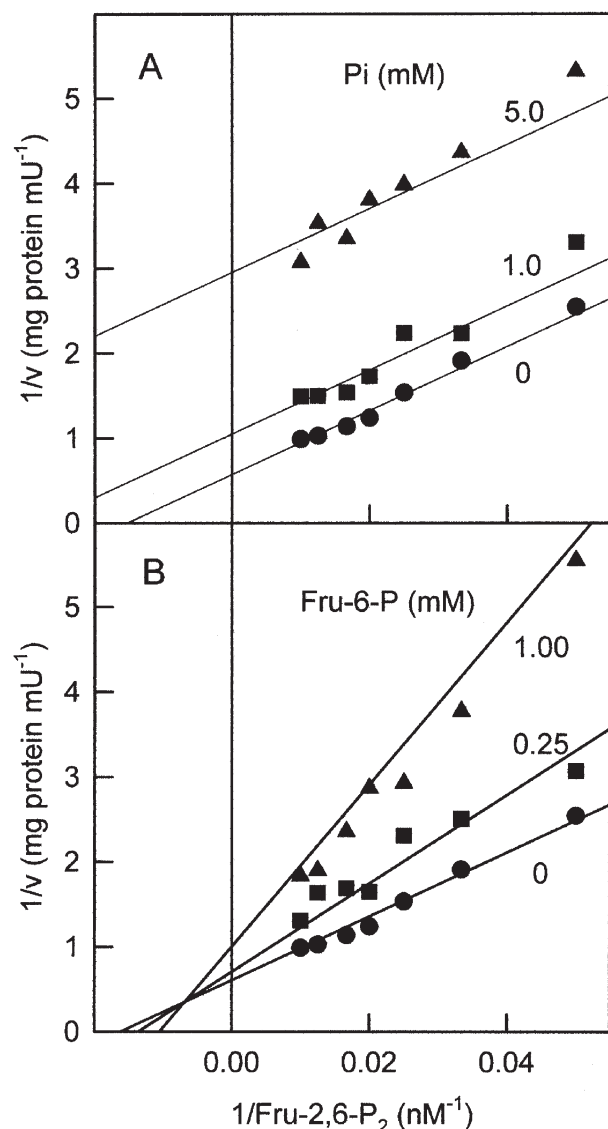
otherwise has no significant homology with any known sequences. In the bifunctional enzyme from other eukaryotes, regions flanking the catalytic domains have a profound influence on the kinetic properties of the enzyme. For example, removal of these regions from the rat liver enzyme decreases  $V_{\max}$  of 6PF2K and its affinity for Fru-6-P, and increases  $V_{\max}$  of Fru-2,6-P<sub>2</sub>ase thus decreasing the activity of 6PF2K relative to that of Fru-2,6-P<sub>2</sub>ase [19]. Furthermore, structural variation in the N- and C-termini, as well as the nature and distribution of phosphorylation sites within these regions, is believed to contribute to the differences between specific isoforms in the properties of the component 6PF2K and Fru-2,6-P<sub>2</sub>ase activities and their response to post-translational modification [7,31,32]. The N-terminal region is likely to serve a comparable regulatory function in plants. Preliminary studies of the recombinant spinach 6PF2K/Fru-2,6-P<sub>2</sub>ase indicate that N-terminal-truncated forms of the enzyme have a much lower activity of 6PF2K relative to Fru-2,6-P<sub>2</sub>ase than the full-length protein studied in this paper (J. E. Markham & N. J. Kruger, unpublished results). Similar differences in the ratio of activities of 6PF2K/Fru-2,6-P<sub>2</sub>ase have been reported for the full-length and truncated proteins from arabidopsis [18]. These observations show that the N-terminal region can influence the component activities of the enzyme and suggest that, by analogy with the mammalian enzyme [7], differences in the N-terminal region (which is less highly conserved than the catalytic core) may be responsible for differences in the regulatory properties of the enzyme between plant species or even tissues.

There is circumstantial evidence to suggest that spinach leaf 6PF2K/Fru-2,6-P<sub>2</sub>ase may be regulated by reversible phosphorylation [33–35]. Analysis of the N-terminal portion of the deduced amino-acid sequence using PHOSPHOBASE [36] suggests 14 potential sites for phosphorylation by calmodulin-dependent protein kinase II and protein kinases A and C. Six of these sites are identified during comparable analyses of the corresponding 6PF2K/Fru-2,6-P<sub>2</sub>ase sequences from arabidopsis and mangrove. Of the four potential phosphorylation sites common to all of these plant sequences, three (Ser138, Ser155, and Ser224 in spinach) yield predictive scores greater than 0.90 during analysis for phosphorylation sites using NetPhos, which exploits a complementary neural network approach [37]. Whether these, or other, residues are phosphorylated *in vivo* remains to be established. Recently, direct evidence has been obtained for phosphorylation of serine residues in 6PF2K/Fru-2,6-P<sub>2</sub>ase in the rosette leaves of arabidopsis [38], although the identity of the specific sites that are modified has yet to be determined.

The kinetic properties of the recombinant 6PF2K/Fru-2,6-P<sub>2</sub>ase are broadly similar to those reported previously for the bifunctional enzyme from spinach leaves [10,11]. The 6PF2K activity of the recombinant protein is activated by  $P_i$  and inhibited by a range of three-carbon phosphate esters and PP<sub>i</sub>. The kinetic constants for Fru-6-P and ATP determined in this paper are consistent with the substrate affinities of the enzyme reported in earlier studies [11]. However, in contrast to previous reports on the partially purified enzyme [8,10], the activity displays standard hyperbolic kinetics with both substrates and there is no evidence for sigmoidal kinetics with respect to Fru-6-P, even in presence of 3-phosphoglycerate. One possible explanation for the apparent sigmoidal kinetics observed by others is contamination of Fru-6-P by  $P_i$ . This would result in a progressive increase in activation by  $P_i$  as the concentration of substrate was increased.



**Figure 6. Influence of  $P_i$  on inhibition of 6PF2K by 3-phosphoglycerate.** Enzyme activity was measured in the presence of 2 mM Fru-6-P, 0.2 mM 3-phosphoglycerate and either 2 mM (●) or 10 mM (○)  $P_i$ . The concentration of ATP was varied as shown. Each value is a single determination of activity based on a four-point time-course of Fru-2,6-P<sub>2</sub> production. Hill coefficients were  $0.89 \pm 0.11$  at 2 mM  $P_i$  and  $0.94 \pm 0.09$  at 10 mM  $P_i$ ; neither of these values was significantly different from unity.



**Figure 7. Inhibition of Fru-2,6- $P_2$ ase by  $P_i$  and Fru-6- $P$ .** Enzyme activity was measured over the range 20–100 nM Fru-2,6- $P_2$  in the presence of  $P_i$  (A) or Fru-6- $P$  (B). The concentration of  $P_i$  was 0 mM ( $\bullet$ ), 1.0 mM ( $\blacksquare$ ), or 5.0 mM ( $\blacktriangle$ ). The concentration of Fru-6- $P$  was 0 mM ( $\bullet$ ), 0.25 mM ( $\blacksquare$ ), or 1.0 mM ( $\blacktriangle$ ). Each value is a single determination of activity based on a four-point timecourse of Fru-2,6- $P_2$  hydrolysis. Hill coefficients were between  $0.92 \pm 0.15$  and  $1.15 \pm 0.29$  in the presence of  $P_i$  (A) and between  $0.85 \pm 0.26$  and  $1.34 \pm 0.29$  in the presence of Fru-6- $P$  (B); none of these values was significantly different from unity. Data are displayed as Lineweaver-Burk plots for presentational purposes only. The lines are the theoretical curves at each concentration of product based on kinetic constants derived from nonlinear regression analysis of the entire data set.

The pronounced activation of 6PF2K by  $P_i$  is due to both an increase in  $V_{\max}^{\text{app}}$  and a decrease in  $K_m^{\text{app}}$  for both of the substrates. This is similar to the effects of  $P_i$  on rat liver 6PF2K/Fru-2,6- $P_2$ ase [27] and consistent with the initial studies on the spinach bifunctional enzyme [10] but contrasts with the apparent decrease in the affinity for ATP during activation by  $P_i$  reported for the purified spinach leaf enzyme [11]. Despite this discrepancy, the 6PF2K activity of

the recombinant enzyme is inhibited by the same range of three-carbon phosphorylated intermediates as that of the enzyme from spinach leaves [8,10,11]. In the present study the effect of 3-phosphoglycerate was to decrease  $V_{\max}^{\text{app}}$  and increase  $K_m^{\text{app}}$  for both Fru-6- $P$  and ATP. The changes in these apparent kinetic parameters are consistent with 3-phosphoglycerate acting as a mixed inhibitor [ $K_{ic} = 0.182 \pm 0.067$  mM,  $K_{iu} = 0.517 \pm 0.133$  mM with respect to ATP;  $K_{ic} = 0.283 \pm 0.104$  mM,  $K_{iu} = 0.421 \pm 0.099$  mM with respect to Fru-6- $P$  (best-fit estimate  $\pm$  SE,  $n = 24$ , calculated from data presented in Figure 5)], although measurements over a greater range of substrate and effector concentrations would be required to establish this relationship. As reported for the enzyme isolated from spinach leaves, the inhibition by 3-phosphoglycerate is reversed by  $P_i$ . In contrast to the corresponding activity of the bifunctional enzyme from rat liver and other mammalian sources [39], 6PF2K is not strongly inhibited by glycerol 3-phosphoglycerate, but is inhibited by  $PP_i$ . The latter effect is consistent with an earlier observation on the enzyme purified from spinach leaves [11].

The relatively high affinity of the Fru-2,6- $P_2$ ase activity of the recombinant enzyme for Fru-2,6- $P_2$  ( $K_m \approx 60$  nM) and the sensitivity of this activity to inhibition by both  $P_i$  and Fru-6- $P$  are comparable to the properties of the bifunctional enzyme isolated from spinach leaves [10,11,15]. Nevertheless, we note that whereas  $P_i$  is a largely uncompetitive inhibitor of the recombinant enzyme, previous studies suggest that it acts competitively even though these reports also claim that  $P_i$  induces sigmoidal kinetics [10] or increases  $V_{\max}$  [11] neither of which is consistent with pure competitive inhibition. Insufficient data are provided in the previous reports to resolve these apparent contradictions.

Irrespective of the minor quantitative differences described above, the kinetic properties of the recombinant 6PF2K/Fru-2,6- $P_2$ ase are in broad agreement with those of the bifunctional enzyme isolated from spinach leaves, and in particular the 90-kDa H-form that has been purified to apparent homogeneity [11]. The affinities of the component activities for their substrates and effectors are within the range of concentrations likely to occur in the cytosol of spinach leaf mesophyll cells (see Table 1 of [26]). This suggests that the levels of these metabolites, which are known to vary throughout the photoperiod, will affect the relative activities of 6PF2K and Fru-2,6- $P_2$ ase thus altering the steady-state level of Fru-2,6- $P_2$  and contribute to the regulation of flux through cytosolic FB-Pase *in vivo*. However, the relative significance of inhibition of 6PF2K activity by 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and dihydroxyacetone phosphate will depend upon the *in vivo* concentration of each of these metabolites and of  $P_i$ , as discussed previously [1].

In conclusion, the kinetic properties of the recombinant enzyme are in agreement with those of the enzyme isolated from spinach leaves. This suggests that the properties of the latter have not been appreciably modified due to proteolysis during extraction. These results corroborate the current view of Fru-2,6- $P_2$  as an internal regulator of sucrose synthesis, integrating the metabolic responses to changes in the relative concentrations of three-carbon phosphate esters, hexose phosphates, and  $P_i$  through allosteric modulation of 6PF2K/Fru-2,6- $P_2$ ase [2].

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